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Therapy with Botulinum Toxin

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Theoretical Analyses of the Functional Regions of the Heavy Chain of Botulinum Neurotoxin

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INTRODUCTION

Background and Rationale

The peptide neurotoxin from *Clostridium botulinum* (botulinum neurotoxin type A; BTX-A) is perhaps the most lethal substance known. On the other hand, its extraordinary selectivity has been exploited in its use as a pharmacological research tool and as an exquisitely potent drug in the treatment of a variety of dystonias and other neurological disorders (1). A number of clinical reports of BTX-A administration (1-3) have emphasized that BTX-A is safe and efficacious in the treatment of some of these conditions, while there is an occasional appearance of relatively benign toxin-induced symptoms. Several studies, however, have noted a low incidence of more serious complications (e.g., mild choking on fluids, distal muscle weakness) (2,4,5), and some patients may need to be considered at risk if subjected to this therapy. The occurrence of these symptoms in some patients suggests that the toxin is already internalized within the peripheral cholinergic nerve endings and is no longer susceptible to nonpenetrating neutralizing antibodies. This situation will become more complex if serotypes other than the now commonly used BTX-A enter the clinical arena. Thus, there is a need to consider a novel therapeutic approach in dealing with the internalized toxin and these adverse reactions. To achieve this goal, a detailed knowledge of the toxin's mechanism of action is required.

At present, seven immunologically distinct serotypes (A-G) have been identified and are the subjects of several extensive reviews (6-9). The primary toxic effect of all the BTX serotypes (excluding the C2 and C3 serotypes [10]) is flaccid paralysis. The toxin prevents the release of acetylcholine in the periphery, thereby blocking neurally induced muscle contraction (11,12). The toxin also inhibits the release or exocytosis of other

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neurally active substances in cholinergic and noncholinergic systems, but at concentrations that are higher than those required to exert effects at peripheral cholinergic synapses.

Like diphtheria toxin (DTX), which inhibits protein synthesis (13), the clostridial neurotoxins (including tetanus toxin [TeTX]) block neurochemical transmission by a series of reactions that is related to their tripartite primary structure. The C-terminal half of the heavy (H) chain binds to ectoacceptors that are predominantly located on the target site, peripheral cholinergic nerve terminals (Fig. 1; step 1), whereupon the entire toxin molecule is internalized by an endocytotic process (step 2). Under low pH conditions the N-terminal half of the H chain is induced to insert into the endocytotic vesicular membrane and form ionic channels. The translocation (step 3) of the toxic light (L) chains from the endocytotic vesicles into the cytoplasmic compartment also occurs under low pH conditions and may depend on the formation and function of the H chain-induced ionic channels. Although the heavy chains of TeTX and DTX are capable of forming functional ionic channels in artificial membranes (14–20), studies with DTX indicate that hydro-

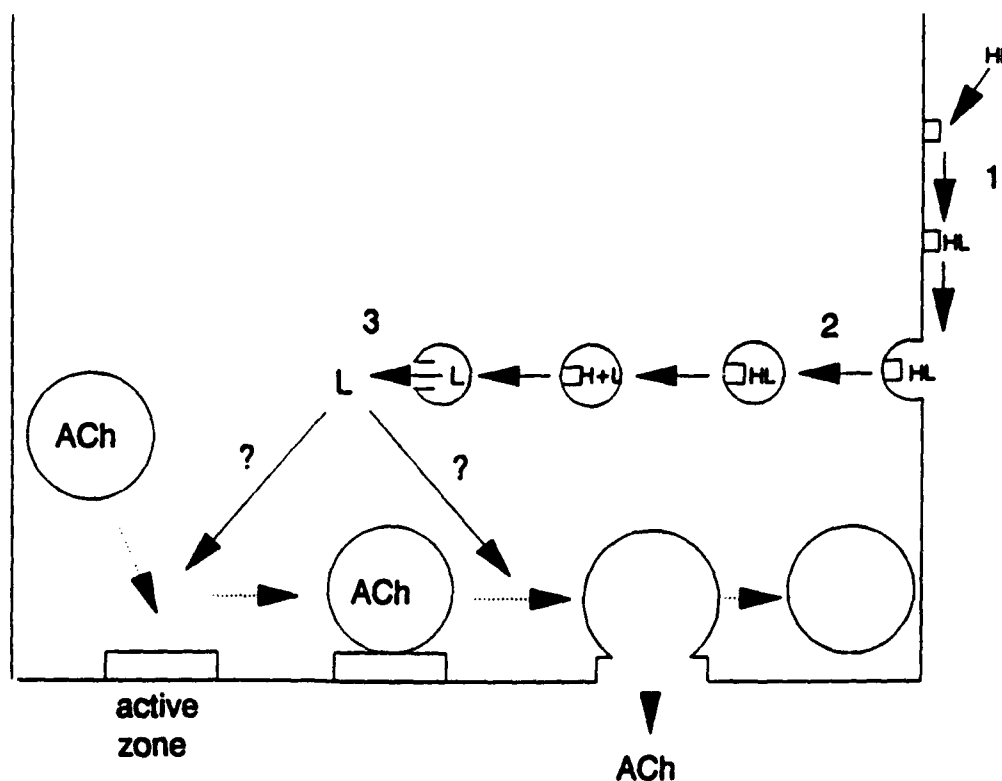


Figure 1 A schematic summary of vesicular release of acetylcholine (ACh) and the hypothetical mechanism of action of botulinum toxin (BTX). The rectangular box represents a cholinergic presynaptic terminal. With depolarizing stimuli, vesicles filled with ACh move toward the active zones and release their contents. The single-chain toxin (HL) binds to ectoacceptors (small squares; step 1) and undergoes endocytosis (step 2), whereupon it separates into heavy (H) and light (L) chains. A decrease in pH in the endocytotic compartment initiates (1) the insertion of the H chain into the endocytotic membrane and the formation of ionic channels and (2) the translocation of the L chain into the cytoplasm (step 3). The final, toxic effect of the L chain is not, as yet, clearly defined, but it is known to differ among the BTX serotypes.

phobic segments of its L chain can also interact with the membrane (21) and take part in the translocation process. Finally, the L chain of BTX exerts the toxic effect, which may be related to its zinc-dependent metalloprotease activity with synaptobrevin-2, a vesicular-associated protein acting as a substrate for some of the serotypes (22). The function of synaptobrevin-2 with regard to vesicular transmitter release is currently not well understood.

If a process in the above multireaction scheme is common to all serotypes, it is possible that a *single* treatment drug could be found and developed for clinical use. From biochemical studies, it appears that at least two different serotype binding sites exist (7,23), which suggests that the ectoacceptor recognition site on the C-terminal halves of the H chains differs among the toxin serotypes. A portion of the present study used a sequence-alignment analysis to determine whether any differences among the primary structures of the serotypes could be detected in this region of the H chain. From electrophysiological data, the mechanism of the neuromuscular blockade is also known to vary among the serotypes (24), and this is discussed by our group in chapter 5.

In contrast to the initial and final steps of the cellular intoxication mechanism, the intermediate L chain translocation (step 3) process, which may involve the H chain-induced ionic channels, is not as well characterized. This is the step that may yield information on a function that the BTX serotypes may have in common. If it is assumed that the ionic channels formed by the toxin H chains play a key role in the L chain translocation, a detailed knowledge of the structure and function of the N-terminal region of the H chains will be of value in understanding the biophysical and pharmacological properties of these ionic channels. In conjunction with the known biophysical properties of toxin-induced channels, our calculations predict that all the BTX serotypes examined have four transmembrane segments located in the N-terminal halves of the H chains that, in turn, could participate in channel formation.

Objectives

A strategy to discover and develop new pharmacological countermeasures against BTX forms the basic thrust of the present research. This study's short-term goal is to identify the amino acid sequences within the H chains of the various serotypes that are involved in the transmembrane regions that make up the toxin-induced ionic channels. Our long-term objective is to identify and develop *single* pharmaceuticals that could be used as pharmacological toxin-induced channel blockers or other medical products for clinical care applications that can counteract the *various* BTX serotypes, rather than develop one for each serotype.

METHODS

Sequence Alignments

Amino acid sequences of the BTX-A, B, C1, D, E, and F serotypes and TeTX were obtained from the National Biomedical Research Foundation (NBRF) data base at the Advanced Scientific Computing Laboratory (National Cancer Institute, Frederick, Maryland). Peptide search, comparison, and analysis software on that facility's mainframe system included FASTA (25), the Sequence Analysis Software Package (Genetics Computer Group, Inc.) (26), the IDEAS package (27), and the programs supported by the Protein Identification Resource (NBRF; Georgetown University Medical Center, Wash-

ington, D.C.). Programs on personal microcomputers included the sequence-alignment program MACAW (Center for Biotechnology Information) (28) and the MacVector analysis package (IBI, New Haven, Connecticut).

Transmembrane Segment Calculations

The Kyte-Doolittle hydrophobic index (29) was calculated using the authors' program and amino acid hydrophobicity scale (i.e., the free energy of transfer of an amino acid from a nonpolar medium to water). This index was calculated as a sum of hydrophobicity values of short peptide segments contained within a consecutively moving window. A second program (GES) was written by us to calculate this free energy of transfer using the amino acid polarity scale, window size, and method described by Engleman *et al.* (30).

Analysis of peptide amphipathicity was performed with the MOMENT program, which uses a consensus scale for the free energy of transfer and a Fourier transform calculation to determine the sequence hydrophobic moment (31-34). Multimeric transmembrane segments were defined on the basis of values for the hydrophobic moment and the hydrophobicity of the sequence of amino acids within a window. The values of the window size (N) for each of these algorithms were selected on the basis of their accuracy in predicting the amino acids within the four transmembrane segments of DTX (35).

RESULTS

Sequence Alignments

Blocks of similar sequences within the toxins examined in this study were determined with MACAW (29) using a modified PAM-120 matrix (36). Regions of amino acid similarity are represented as the larger blocks in Fig. 2. Gaps were automatically inserted by this program to optimize the alignment, and the residue positions are displayed as relative locations on the H chain. The relative amino acid positions for the channel-forming region range from 139 to 229 for BTX-A (see Fig. 3 and "Hydrophobic Analyses" and "Hydrophobic Moment Analysis," below). While the ectoacceptor-recognition sites are not, as yet, clearly defined, it is notable that a large region of dissimilar sequences among the serotypes occurs in the C-terminals of the H chains (relative positions: 687-843). From our initial analysis it is anticipated that the experimentally observed differences among the BTX serotype binding properties will be related to the different sequences occurring within this range.

Hydrophobic Analyses

Our approach in localizing channel-forming regions was to use several different computer-assisted calculations to determine free energies of peptide insertion into biological membranes. We assumed that channels are composed of amphipathic sequences that contain both hydrophobic and hydrophilic amino acid residues (32,33,37). The KD algorithm and amino acid hydrophobicity scale developed by Kyte and Doolittle (29) were used in this portion of the study to obtain an initial estimate of the number of possible transmembrane segments in clostridial toxin H chains. The horizontal bars in Fig. 3 show the extent of the two calculated hydrophobic regions in the N-terminal half of the BTX-A H chain that are long enough to span a biological membrane as either an α -helix or a β -strand. Transmembrane β -strands require at least 10 amino acid residues, while trans-

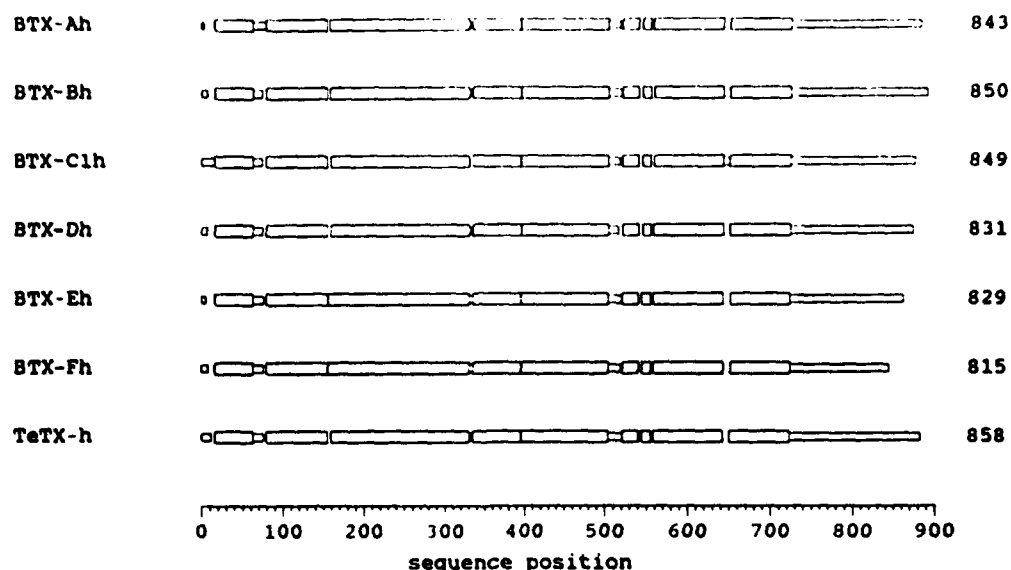


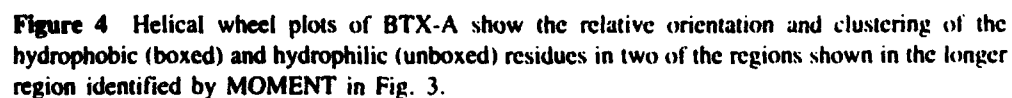
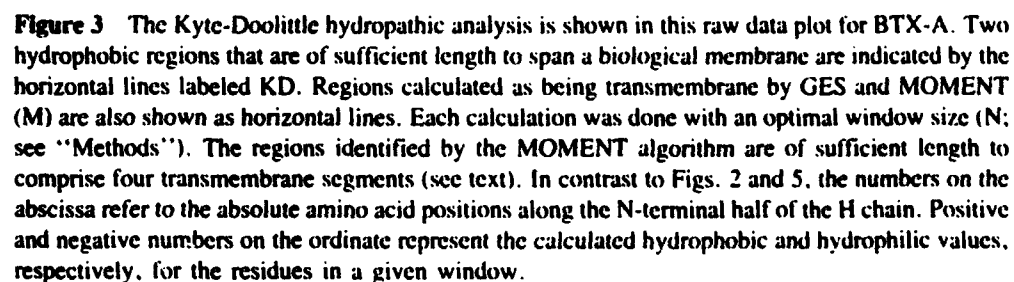
Figure 2 A schematic alignment of the primary structures of the heavy (H) chains of BTX-A, B, C1, D, E, and F and TTX. The larger rectangles represent regions of similar amino acid sequences. The N-terminal end is on the left and the C-terminal end of the H chain is on the right. Gaps were optimally inserted by MACAW. The residue positions are relative to their location on the H chain.

membrane α -helices need about 20 residues (38). The output from the GES program (Fig. 3) indicates that there were also two predicted membrane insertion regions having a sufficient number of amino acids. Although the detected hydrophobic segments were predicted to be transmembrane regions, neither the KD nor the GES algorithm can determine whether they are α -helices or β -strands. From the MACAW (Fig. 2) and the transmembrane segment analyses (Fig. 3), similar results were obtained with the other serotypes examined, and this is consistent with the idea that these ionic channels have similar properties.

Hydrophobic Moment Analysis

Using the Eisenberg algorithm and consensus scale, two regions in the BTX-A H chain were predicted by the MOMENT program to be amphipathic. Because of extensive length of the second segment, it is suggested that it could represent three separate regions. Thus, a total of four transmembrane segments could form in this portion of the H chain. It should be noted here that the MOMENT analysis was quantitatively more accurate than the KD or GES programs when searching for the four putative transmembrane α -helices of DTX (see "Methods," above) and was thus operationally defined as the best algorithm in the present search for the transmembrane segments in the BTX serotypes.

Although the low values of the sequence hydrophobic moment for BTX could not be categorized as strongly reflecting either amphipathic α -helices or β -strands in these regions, an α -helical arrangement for these segments was tentatively assumed. One possible configuration of these amphipathic regions was visualized by the helical wheel display (39). Plots shown in Fig. 4 illustrate the relative orientation of hydrophobic (boxed) and hydrophilic (unboxed) residues within the regions identified in the MOMENT



analysis. The hydrophilic residues tended to be clustered within one quarter of a possible α -helical conformation, an orientation that will be referred to below (see "Discussion").

Additional Sequence Alignments

A result from another set of sequence comparisons is illustrated in Fig. 5. Amino acid residues in the predicted amphipathic regions of BTX-A, C1, and D and TeTX are similar to those in the putative transmembrane S2 and S3 segments of voltage-gated potassium channels in vertebrate and invertebrate neurons (40). The apparent similarity between peptide toxins and voltage-gated potassium channels in this region lends plausible indirect evidence in support of our assumption (see "Sequence Alignments," above) that the predicted amphipathic regions in these toxins are involved in the formation of ionic channels.

DISCUSSION

The main goals of this study were to examine the ectoacceptor-recognition region (C-terminal half) and to localize the amino acid residues involved in the formation of ionic channels (N-terminal half) within the H chains of various BTX serotypes and TeTX.

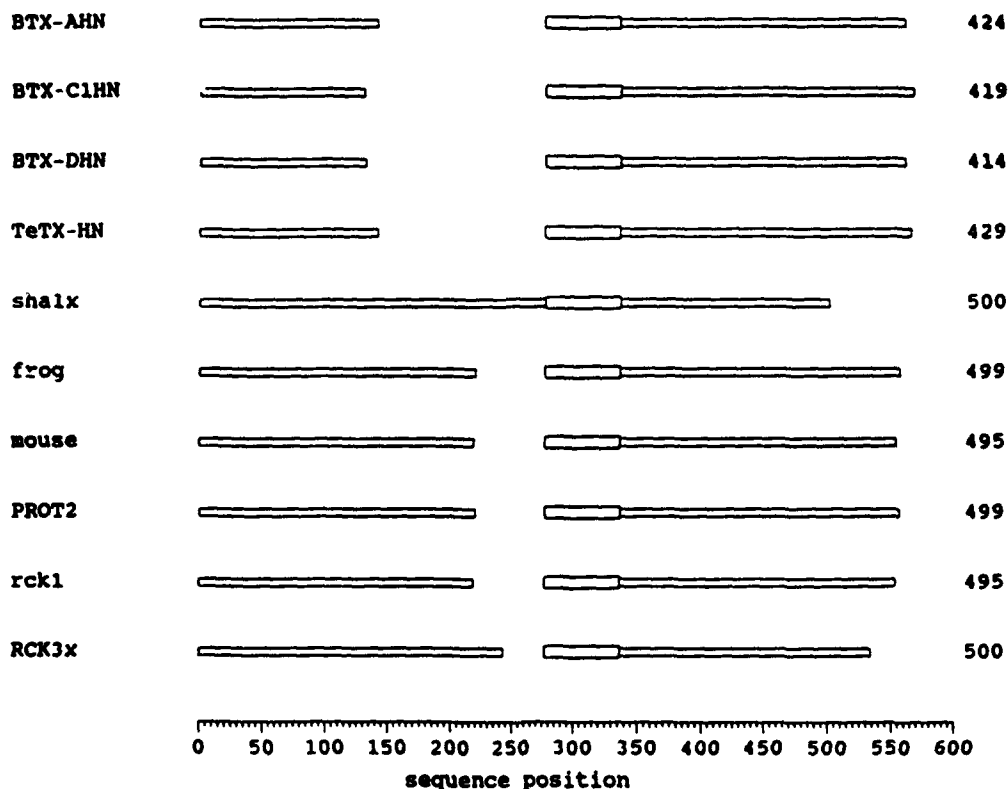


Figure 5 Sequences of amino acid residues in the hydrophobic regions of BTX-A, C1, and D and TeTX are similar to those of transmembrane segments S2 and S3 of peptides forming voltage-gated potassium channels from a variety of species. Only the N-terminal halves of the H chains (HN) are illustrated. As in Fig. 2, the residue positions are relative to their location on the H chain.

Single large regions within the C-terminal halves were not similar according to our alignment analysis. This is consistent with the findings of others that the BTX-A and B serotypes do not compete with the same ectoacceptor (23). It is of interest that two different lectins have been reported to prevent six different BTX serotypes, as well as TeTX, from binding to rat brain membranes (41). These authors suggested that while the serotype ectoacceptors may not be identical, they may share certain structural features, such as being sialoglycoproteins. Thus, a lectin may qualify as a universal antagonist acting extracellularly against all the toxin serotypes—a result that may be clinically relevant in protecting individuals before BTX exposure.

Unlike the region of dissimilarity in the C-terminal halves, the amino acid sequences in the N-terminal halves of the H chains of these serotypes were similar and were predicted to have four segments (Fig. 2). Experiments with TeTX H chain fragments (42) showed that the N-terminal portion of the H chain is involved in a pH-dependent translocation process, and according to Binz et al. (43), this region includes the amphipathic segments as defined by our analyses.

The schematic diagrams in Fig. 6 may be used as a starting point in portraying the formation of ionic channels by toxin H chains. If only a relatively short portion of the H chain traverses the membrane to form two antiparallel segments, it would be expected that both the N- and the C-terminals would remain on the *cis* side of the membrane (Fig. 6a-c). The steps for binding (Fig. 6a), insertion (Fig. 6b), and alignment (Fig. 6c) are shown from a view lateral to the membrane (parallel lines). Further details in the alignment process are illustrated in the view normal to the membrane (Fig. 6d). Shaded areas that correspond to the hydrophilic residues depicted in the helical wheel plots (Fig. 4) are aligned toward the hydrophilic residues located within the opposite region. From the biophysical evidence the concentration-response relation for DTX and BTX is a power function (15,18), a result suggesting that there must be at least two toxin molecules involved in producing a conducting channel. The dimeric arrangement in Fig. 6e is based

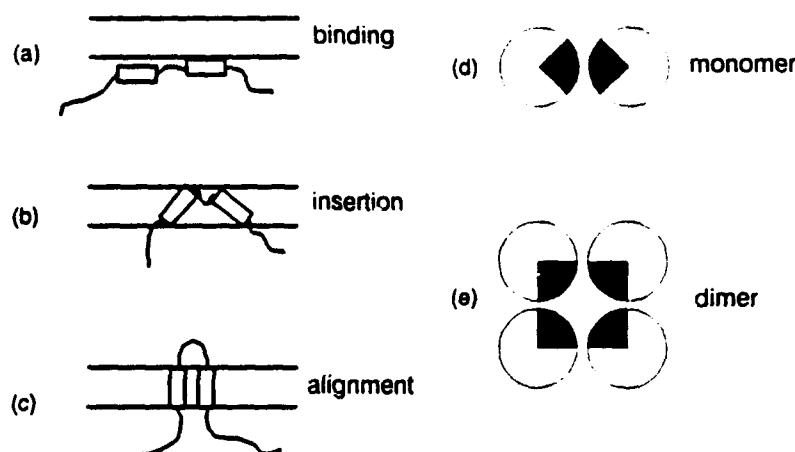


Figure 6 In this hypothetical scheme, two amphipathic regions (rectangles) are shown to bind (a), insert (b), and align (c), themselves starting on the *cis* side of the membrane (parallel lines in panels a-c). The resulting transmembrane topology predicts that both the N- and the C-terminals of the toxin H chain are located on the *cis* side. The top view (d) shows how the hydrophilic residues (shaded areas) could be aligned. (e) A dimer forms a membrane-embedded, predominantly hydrophobic structure whose center is surrounded by the side chains of the hydrophilic residues.

on the hypothesized topology of peptides that form voltage- (44,45) and agonist-gated channels (46,47). These segments are depicted as having residues with hydrophilic side chains that line an aqueous core, interleaved between residues with hydrophobic side chains that face toward the lipid environment (48). This arrangement is consistent with the predicted orientation of tetramers of synthetic fragments of the TeTX H chain that form functional ionic channels (49).

Finally, the alignments of voltage-gated potassium and toxin-induced channels shown in Fig. 5 raise the question of whether their pharmacological properties are similar. Indeed, tetra-alkylammonium derivatives have been demonstrated to block anthrax-induced channels (50) in a manner similar to tetraethylammonium, a well-known blocker of neuronal potassium channels.

From these similarities in primary structures and predicted transmembrane segments, we hypothesize that the BTX-induced ionic channels represent regions of similar amino acid sequences that are common to all of the BTX serotypes and that these structures could be focal points in the future development of therapeutic drugs. Rather than developing drugs that are unique for each serotype (because of their different antigenic, binding, and toxic properties), it is proposed that a *single* drug could be developed that would block the ionic channels formed by the *various* BTX serotypes. If functional toxin-induced channels are necessary for the translocation of the L chain, this single drug may be an effective therapy against the clinical complications produced by all of the BTX serotypes.

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